Simplifying Fatty Acid Analyses in Multicomponent Foods with a Standard Set of Isothermal GLC Conditions Coupled with ECL Determinations

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Abstract

It is common to use some sort of temperature program in the analysis of fatty acid methyl esters. For samples that contain only a few components or in situations where all samples are basically the same, temperature programming can offer certain advantages. Unfortunately, samples such as these are certainly not the norm in routine food fatty acid analysis. A gas–liquid chromatographic column, the Carbowax-20M fused-silica phase (cross-linked) recommended by Ackman as a "standard" for fatty acid analysis (it simplifies the work and reduces the chance for errors), is used to chromatograph mixtures of methyl esters at column temperature settings that range from 170°C to 250°C. It is determined that three specific temperatures (183°C, 212°C, and 247°C) permit complete resolution of a wide array of acids under strictly isothermal conditions. Equivalent chain length values have been compiled for use in assigning identities of fatty acid methyl esters. Also, the specific responses of individual methyl esters to alterations in column temperature can be of use in the identification of peaks in complex mixtures.

Introduction

Gas–liquid chromatography (GLC) of fatty acid methyl esters (FAMEs) plays an important role in the analysis of the fatty acid composition of foods; it is used to quantify the individual acids once they have been converted to volatile derivatives (methyl esters in this case), separated, and identified in the chromatographic process. In undertaking the present work, the author hoped to contribute in some way to the evolution and acceptance of more standardized and efficient procedures for the quantitation of FAMEs, particularly in complicated food mixtures. The fatty acid contents of mixtures, for example, in recipes, entrees, and diet-composites, have been of special interest recently to nutritional scientists and food technologists; it has often been difficult to achieve consistently good results in fatty acid analysis because of the great variety of ingredients (e.g., plant, animal; dairy; fresh water, marine) that go into the formulation of these mixtures.

The substantial body of literature published by Ackman (references 1 and 2 and those cited therein, for example) is a rich source of information and ideas. Of special significance are the reports on the usefulness of one particular type of GLC column, the Carbowax-20M liquid phase, in cross-linked form, on a flexible fused-silica capillary. Ackman has discussed its benefits and has urged other researchers to at least consider it as a "standard" column for interlaboratory studies and for general fatty acid work (1–3); such a column was used for the analysis of FAMEs in the present study.

Also critical to the conduct of the present study was Ackman's report on the analysis of a marine oil and a canola oil on the Carbowax-20M GLC column (1); in a detailed description of the analysis of these natural products, he demonstrated how useful they are as secondary standards. Joseph and Ackman followed up on this idea by offering to make available, upon request, capsules of a menhaden oil referred to in the official methods they developed for determination of the fatty acid composition of marine oils (AOAC Method 991.39 [4,5] and AOCS Method Ce-1b-89 [6,7]).

At the outset, after perusing relevant literature and drawing from personal experience, it was apparent that GLC retention data (for FAMEs) could be conveniently managed and communicated if it were expressed in terms of the equivalent chain length (ECL) (8–15). Consequently, a commitment to the routine use of ECL descriptors became another key ingredient in the development of the strategy designed to standardize and simplify the collection and management of fatty acid compositional information.

The principle cornerstone of the protocol that evolved is the reliance on only a few selected column temperatures for the analysis of complicated FAME mixtures and avoidance of temperature programming so far as possible. These "preferred" temperatures were carefully chosen, as will be described, to minimize the occurrence of coincident peaks during isothermal chromatography. Temperature programming will be useful in special situations, and this powerful tool will be used in this laboratory, as it has been in the past (16–20), but only under special circumstances, such as when the identity of all FAME components are known beforehand and all samples in a series are of essentially the same composition. In those few instances in which

temperature programming is unavoidable, a Carbowax-20M liquid-phase column can reduce errors because, as pointed out by Ackman (2), the elution order will hardly change on going from isothermal operations to temperature programming with the use of that phase.

Four additional practices were adopted as standard operating procedure as a result of the experiments described herein: (*a*) chromatography was performed with the Carbowax-20M liquid-phase column to avoid problems with chain-length overlap and to reduce errors in general fatty acid work (1–3,21–23); (*b*) ECL values were computed for each FAME in a report of analysis; (*c*) secondary standards consisting of marine oil and canola oil were used at a minimum for quality control and retention measurements (1,2,4,6); and (*d*) chromatography was performed with hydrogen as the carrier gas not only to save time but also to extend the range of linear velocities that can be used in response to the complexity of the samples without serious diminution of column resolution (2,21,24–28).

The task of summarizing available data on fatty acid contents for inclusion into databases of food composition is a daunting task to begin with (29,30); however, this work is made even more difficult because of the multitude of GLC methods that are being used (in other words, different columns and different temperature programs). Ackman has emphasized this in a recent review in which he states that the main problem in identification of peaks for fatty acids is the increased use of both programming and high-polarity columns (2). This heterogeneity of method and practice hinders the applicability of literature data. The work described herein was initially undertaken to try to bring more order to the process; as a result, assembled in this report are ECL values for a great many of the fatty acids, if not all, that might be encountered in the analysis of common food materials and their mixtures.

Ackman reported this information for a column temperature of 180°C (1); the information present herein simply extends his tables to include information on the dependence of ECL on column temperature. The information has been organized both in tabular form and graphically to help the analyst select the best column temperatures according to the nature of the samples under study and to facilitate comparison with other relatively recent reports that provide ECL information and utilize similar chromatography columns (1,31–34).

Experimental

Lipid standards

Several secondary standards were purchased from commercial suppliers: PUFA-1, a mixture of FAMEs derived from a marine source (Matreya, Pleasant Gap, PA); PUFA-2, FAMEs from land sources (Matreya); and Mix 401, a mixture of FAMEs (Supelco, Bellefonte, PA). Capsules of fish oil, the steam-deodorized menhaden oil used in collaborative studies that led to AOAC and AOCS official methods (4–7), were provided by Gloria Seaborn and Jeanne Joseph of the National Marine Fisheries Service (Charleston, SC). Canola oil was purchased from a local market. Various individual triacylglycerols and FAMEs were purchased from Nu-Chek Prep (Elysian, MN).

Gas–liquid chromatography

Instrumentation and columns.

A Hewlett-Packard (San Fernando, CA) Model 5840 and a Siemens USA (Alpharetta, GA) SICHROMAT 2-8, both equipped with a flame-ionization detector (FID) and a split/splitless capillary inlet system, were used interchangeably for GLC. The GLC columns were wall-coated open-tubular (WCOT) columns made of fused-silica capillaries. The dimensions were 60 m × 0.25-mm i.d., with a 0.25-µm film thickness of polyethylene glycol liquid phase (STABILWAX, a cross-bonded Carbowax-20M, cat. #10626, Restek, Bellefonte, PA). The injector temperatures were 300°C, and the detector temperatures were 320°C. The carrier gas was hydrogen; it was adjusted to a linear velocity of 40 cm/s at a column temperature of 212°C. The septum purge flow rate was 2 mL/min.

Capillary injection liners

The inlet systems in both instruments were used in a split mode with a split ratio of approximately 100:1. Both were fitted with deactivated inlet sleeves (cup splitter type) purchased from Restek (cat. #20709 for the Hewlett-Packard chromatograph and #20825 for Siemens chromatograph). Prior to installation, the sleeves had been packed with approximately 0.5 cm of GLC packing (1% JXR on Gas Chrom Q) that was held in place by a small plug of deactivated fused-silica wool (Restek, cat. #20790) placed above and below the packing.

Injections of sample were made by hand-injection with a Hamilton 7101, 10-µL syringe. Injection volumes were generally 1–4 µL of FAME sample, sandwiched between 1 µL of isooctane behind the sample and 1 µL ahead of the sample (2).

Purification of carrier gas

Even small amounts of oxygen and water are deleterious to GLC columns. Zero-grade gas (99.997%) was purchased and then further treated to remove contaminants with the following series of traps: charcoal for hydrocarbons (cat. #2-2446M, Supelco); molecular sieve for water (cat. #20685, Restek); and indicating trap for oxygen and water (cat. #20623, Restek).

Computing

Software

A commercially available spreadsheet program (Excel, Ver. 4, Microsoft, Redmond, WA) was used for data processing. Retention times were entered manually with a standard keyboard. The spreadsheet was programmed by the author using Excel's standard commands and functions.

Hardware

The computer was a Macintosh IIci with 5 mB of memory and an 80 mB hard drive (Apple Computer, Cupertino, CA).

Results and Discussion

Creation of a database of ECL values for various temperatures

Mixtures of FAME (standards and secondary standards) were analyzed at column temperatures that ranged from 170°C to

250°C, and retention times were processed with the spreadsheet program. All measurements of retention times were those reported according to the chromatograph and represent the time from injection of the sample to the apex of the peaks. Ackman's method (1), which involves measuring to the intercept of the peak frontal tangent with the baseline, would provide more precise retention times for research samples, but it was impractical as a routine practice in the context of routine analysis, at least in this laboratory as it is currently equipped. The reader is directed to a report by Bannon and co-workers (reference 31 and those cited therein) for thorough coverage on errors in ECL measurements that arise from peak overlap, column overload, and other sources.

The first step in data processing was to convert retention time (t) for each FAME into corrected retention time (t') by subtraction of holdup time (t_M). t_M was calculated for each chromatographic run as described by Bannon and co-workers (31) with the following equation:

$$t_M = \frac{t_{18:0}^2 - (t_{16:0}\, t_{20:0})}{2t_{18:0} - t_{16:0} - t_{20:0}} \qquad \text{Eq 1}$$

where $t_{16:0}$, $t_{18:0}$, $t_{20:0}$ are the retention times for the methyl esters of 16:0, 18:0, and 20:0, respectively. The spreadsheet was programmed to develop a linear regression for the relation between $\log_{10}(t')$ and the number of carbon atoms for three saturated FAMEs, which were selected by the operator so that they "bracket" the FAME of interest on the GLC trace (Table I). The resulting linear regression equation was then used to calculate ECL for each peak in that particular chromatogram. This processing was repeated for all chromatograms made at the various temperatures. The ECL results for all FAME components, at all temperatures, were organized into a single database for further study.

A shorthand notation of the form $M{:}N\omega X$ has been used to denote fatty acid structure. M signifies the chain length (number of carbon atoms), N is the total number of ethylenic bonds, and X (when ωX is appended) is the position of the ethylenic bonds with respect to the penultimate carbon (i.e., from the terminal methyl group). Unless otherwise indicated, all double bonds are assumed to be *cis* geometry, and methylene interrupted in the cases where the fatty acid is polyolefinic. According to this form, ωX means the same as n-X or simply nX; for example, α-linolenic acid, 18:3ω3, 18:3n3, and 18:3n-3 all refer to the same chemical entity.

Prediction of ECL for a FAME at a specific temperature

The relationship between column temperature and ECL for individual FAMEs was examined and quantitated with the use of regression analysis as previously described. The slope (m) and y-intercept (b) coefficients for the least-squares line developed for each FAME are given in Table I, along with coefficients of determination (R^2). ECL values were readily obtained for any of the FAMEs, at a particular column temperature, by using m and b in Equation 2. For example, if we use the regression line developed from the corrected retentions for three saturated FAMEs (16:0, 18:0, and 20:0) in the same chromatogram, the ECL for 18:3ω6 at 212°C becomes:

$$ECL_{16,18,20}^{212°} = (212m) + b =$$
$$(212 \times 0.003025) + 18.410 = 19.051 \qquad \text{Eq 2}$$

The straight-line model describes the relationship between ECL and temperature well, as can be seen in Figure 1 where the lines for several FAMEs are superimposed over their individual ECL values. The R^2 show the strength of this linear relation as well, since it was very close to unity for most of the FAMEs, with the exception of branch-chain acids (Table I). In Figure 1, the ECL of 24:0 does not change with alterations in column temperature, as expected by definition. For unsaturated FAMEs, however, ECL responds to a change in column temperature (Δ_{ECL}) in a manner that is directly proportional to temperature, and the response becomes more pronounced with the addition of more ethylenic bonds (i.e., Δ_{ECL} for 24:1ω9 << Δ_{ECL} for 22:5ω3 < Δ_{ECL} for 22:6ω3) (Figure 1).

Visual inspection of the regression lines (ECL versus temperature) for the few branch-chain FAMEs for which data were collected clearly show that they are linear also (graphs not included); this is not apparent when considering only the numerical value for R^2 in the absence of a graphical representation of the data. One unfortunate characteristic of regression analysis is that it will indicate that only a weak linear relationship exists (i.e., $R^2 \approx 0$) if the slope of the line is very small, when in fact a fairly strong linear relationship does exist, as is the case with branched-chain FAMEs. This weakness in regression analysis may have led other researchers to the conclusion that branched FAMEs do not behave in the same predictable fashion as other FAMEs (35).

Small differences traceable to the age (history) of the column

On examination of ECL results and dates of chromatography, it appeared that the column had undergone a shift in polarity (a slight increase) during its first several days of use. This first became apparent in the distribution of points in displays like in Figure 1, in which it looked as if there were two distinct populations of values for each unsaturated FAME. This can be observed more easily in Figure 2 where solid lines represent the column when it was new (first several days) and broken lines represent the same column after a period of use at 180°C that lasted approximately two weeks. Except for the initial shift in apparent polarity during the first few days, there was no evidence of any further change as the column aged over the course of the study (approximately 3 months). Fortunately, the differences observed between "fresh" and "aged" columns in this study were considerably smaller than those observed by Jamieson (11) in his work with packed columns. Ackman (3,21) and Joseph and Ackman (4) have already reported that such high levels of consistency, over the life of a column, are an important benefit of columns of that particular type.

Selecting temperatures to avoid coincident peaks

Overlapping peaks have been an aggravating problem in the analyses of complex and varied mixtures, so much so that these difficulties prompted the start of the present investigation. As an example, consider four acids (22:6ω3, 24:1ω9, 22:5ω3, and 24:0)

Table I.

Fatty acid*	ECL values Preferred temperatures explained in the text 183°	212°	247°	Regression analysis: ECL vs temperature Sat'd FAME Ref†	Coefficients‡ Slope $m \times 10^3$	Intercept b	R^2	ECL values Compare with published values§ 175°	180°	195°	200°
14:0	14.000	14.000	14.000	—	— By definition —		—	14.000	14.000	14.000	14.000
4,8,12-TMTD	14.052	14.037	14.019	14,16,18	-0.522	14.148	.899	14.057	14.054	14.046	14.044
14:1ω9	14.175	14.218	14.270	14,16,18	1.491	13.902	.950	14.163	14.170	14.193	14.200
14:1ω7	14.281	14.319	14.364	14,16,18	1.286	14.046	.945	14.271	14.277	14.297	14.303
14:1ω5	14.398	14.435	14.479	14,16,18	1.268	14.166	.878	14.388	14.394	14.413	14.420
Iso-15:0	14.512	14.514	14.517	14,16,18	0.082	14.497	.074	14.511	14.512	14.513	14.513
Anteiso-15:0	14.667	14.680	14.695	14,16,18	0.447	14.585	.527	14.663	14.665	14.672	14.674
14:1?	14.743	14.772	14.807	14,16,18	1.003	14.559	.917	14.735	14.740	14.755	14.760
15:0	15.000	15.000	15.000	—	— By definition —		—	15.000	15.000	15.000	15.000
15:1ω8	15.272	15.314	15.365	14,16,18	1.452	15.006	.934	15.260	15.267	15.289	15.296
Iso-16:0	15.508	15.506	15.503	14,16,18	-0.082	15.523	.034	15.509	15.508	15.507	15.507
Pristanic	15.741	15.702	15.656	14,16,18	-1.328	15.984	.862	15.752	15.745	15.725	15.718
16:0	16:000	16:000	16:000	—	— By definition —		—	16:000	16:000	16:000	16:000
16:1ω9	16.139	16.187	16.245	16,18,20	1.659	15.835	.979	16.125	16.134	16.159	16.167
16:1ω7	16.287	16.325	16.372	16_I 18,20	1.323	16.045	.952	16.277	16.283	16.303	16.310
16:1ω5	16.395	16.433	16.479	16,18,20	1.310	16.155	.959	16.384	16.391	16.410	16.417
trans-16:1ω13	16.485	16.561	16.654	16,18,20	2.639	16.002	.960	16.464	16.477	16.517	16.530
Iso-17:0	16.510	16.511	16.512	16,18,20	0.028	16.505	.017	16.510	16.510	16.510	16.511
16:2ω7	16.635	16.725	16.833	16,18,20	3.088	16.070	.984	16.610	16.626	16.672	16.688
Anteiso-17:0	16.668	16.680	16.695	16,18,20	0.420	16.591	.705	16.665	16.667	16.673	16.675
16:2ω4	16.861	16.922	16.995	16,18,20	2.079	16.481	.984	16.845	16.855	16.886	16.897
Phytanic	16.918	16.881	16.837	16,18,20	-1.263	17.149	.618	16.928	16.922	16.903	16.897
17:0	17.000	17.000	17.000	—	— By definition —		—	17.000	17.000	17.000	17.000
16:3ω4	17.200	17.279	17.374	16,18,20	2.722	16.702	.993	17.178	17.192	17.233	17.246
17:1ω8	17.220	17.269	17.328	16,18,20	1.692	16.910	.905	17.206	17.215	17.240	17.248
Iso-18:0	17.483	17.485	17.488	16,18,20	0.082	17.468	.079	17.482	17.483	17.484	17.484
16:4ω3	17.601	17.694	17.805	16,18,20	3.182	17.019	.993	17.576	17.592	17.639	17.655
16:4ω1	17.763	17.854	17.964	16,18,20	3.132	17.190	.993	17.738	17.754	17.801	17.816
18:0	18.000	18.000	18.000	—	— By definition —		—	18.000	18.000	18.000	18.000
18:1ω13	18.120	18.170	18.229	16,18,20	1.702	17.809	.995	18.107	18.115	18.141	18.149
18:1ω9	18.212	18.253	18.303	16,18,20	1.435	17.949	.978	18.200	18.207	18.229	18.236
18:1ω7	18.277	18.318	18.367	16,18,20	1.396	18.022	.983	18.266	18.273	18.294	18.301
18:1ω5	18.401	18.438	18.483	16,18,20	1.274	18.168	.981	18.391	18.397	18.416	18.423
18:2ω6	18.660	18.727	18.808	16,18,20	2.302	18.239	.995	18.642	18.653	18.688	18.699
18:2ω4	18.862	18.924	18.999	16,18,20	2.151	18.468	.995	18.844	18.855	18.887	18.898

(*continued on page 499*)

* The list of fatty acid designations in this column was intended to be consistent with the list published by R.G. Ackman (reference 1, cf. tables 4.3A and 4.3B with this table). With the exception of FAMEs 22:2I and 22:2J, the shorthand notation used to identify the fatty acids is described in the text. Ackman has determined that 22:2I and 22:2J are C_{22} non-methylene-interrupted dienoic acids peculiar to marine oils and lipids (reference 1 and references contained therein).

† Within each row, ECL calculations were based on the retentions of the three saturated FAMEs identified in this column using the notation n_1,n_2, and n_3 which refer to the number of carbons in the fatty acid backbone. For example, "14,16,18" would indicate that 14:0, 16:0 and 18:0 were the FAMEs used to determine ECL.

‡ The values for m and b are used for prediction of the ECL for FAMEs at a specific temperature, using the equation of a straight line, as shown in Equation 2.

§ These temperatures are listed to make it convenient to compare ECL values with those of Christie (32) (cf. 175°), Ackman (1) (cf. 180°), Kramer et al. (33) (cf. 195°), and Bannon et al. (31) (cf. 200°).

Table I. (*continued from page 498*)

Fatty acid*	ECL values Preferred temperatures explained in the text 183°	212°	247°	Regression analysis: ECL vs temperature Sat'd FAME Ref†	Coefficients‡ Slope $m \times 10^3$	Intercept b	R^2	ECL values Compare with published values§ 175°	180°	195°	200°
18:3ω6	18.964	19.051	19.157	16,18,20	3.025	18.410	.997	18.939	18.955	19.000	19.015
19:0	19.000	19.000	19.000	—	— By definition —		—	19.000	19.000	19.000	19.000
18:3ω3	19.298	19.382	19.483	16,18,20	2.882	18.771	.996	19.275	19.290	19.333	19.347
18:4ω3	19.602	19.706	19.832	16,18,20	3.590	18.945	.996	19.573	19.591	19.645	19.663
18:4ω1	19.718	19.818	19.938	16,18,20	3.447	19.087	.994	19.690	19.707	19.759	19.776
20:0	20.000	20.000	20.000	—	— By definition —		—	20.000	20.000	20.000	20.000
20:1ω15	20.118	20.173	20.238	16,18,20	1.876	19.775	.985	20.103	20.113	20.141	20.150
20:1ω12	20.123	20.189	20.268	16,18,20	2.263	19.709	.897	20.105	20.116	20.150	20.162
20:1ω11	20.130	20.199	20.283	16,18,20	2.379	19.695	.888	20.111	20.123	20.159	20.171
20:1ω9	20.187	20.234	20.290	16,18,20	1.613	19.892	.982	20.174	20.182	20.207	20.215
20:1ω7	20.267	20.310	20.361	16,18,20	1.479	19.996	.986	20.255	20.262	20.284	20.292
20:2ω6	20.646	20.715	20.799	18,20,22	2.391	20.208	.996	20.626	20.638	20.674	20.686
20:3ω6	20.901	20.999	21.117	18,20,22	3.363	20.286	.997	20.875	20.891	20.942	20.959
21:0	21.000	21.000	21.000	—	— By definition —		—	21.000	21.000	21.000	21.000
20:4ω6	21.113	21.236	21.386	18,20,22	4.266	20.332	.998	21.079	21.100	21.164	21.185
20:3ω3	21.286	21.373	21.478	18,20,22	2.996	20.738	.997	21.262	21.277	21.322	21.337
20:4ω3	21.537	21.653	21.793	18,20,22	3.991	20.807	.997	21.505	21.525	21.585	21.605
20:5ω3	21.756	21.896	22.065	18,20,22	4.830	20.872	.998	21.717	21.741	21.814	21.838
22:0	22.000	22.000	22.000	—	— By definition —		—	22.000	22.000	22.000	22.000
22:1ω11	22.109	22.164	22.230	18,20,22	1.891	21.763	.992	22.094	22.103	22.132	22.141
22:1ω9	22.170	22.221	22.281	18,20,22	1.734	21.853	.993	22.156	22.165	22.191	22.200
22:2I	22.263	22.308	22.361	18,20,22	1.531	21.983	.995	22.251	22.259	22.282	22.289
22:2J	22.371	22.465	22.577	18,20,22	3.220	21.782	.995	22.346	22.362	22.410	22.426
22:2ω6	22.639	22.711	22.797	20,22,24	2.467	22.188	.997	22.620	22.632	22.669	22.681
21:5ω3	22.799	22.937	23.103	20,22,24	4.739	21.932	.994	22.761	22.785	22.856	22.880
22:3ω6	22.882	23.002	23.146	20,22,24	4.125	22.128	est	22.849	22.870	22.932	22.953
23:0	23.000	23.000	23.000	—	— By definition —		—	23.000	23.000	23.000	23.000
22:4ω6	23.106	23.233	23.386	20,22,24	4.382	22.304	.996	23.071	23.093	23.158	23.180
22:3ω3	23.284	23.373	23.481	20,22,24	3.067	22.723	.996	23.260	23.275	23.321	23.336
22:5ω6	23.383	23.510	23.663	20,22,24	4.375	22.583	est	23.348	23.370	23.436	23.458
22:4ω3	23.533	23.656	23.805	20,22,24	4.250	22.755	est	23.499	23.520	23.584	23.605
22:5ω3	23.747	23.892	24.067	20,22,24	5.010	22.830	.997	23.707	23.732	23.807	23.832
24:0	24.000	24.000	24.000	—	— By definition —		—	24.000	24.000	24.000	24.000
22:6ω3	24.028	24.184	24.373	20,22,24	5.391	23.041	.997	23.984	24.011	24.092	24.119
24:1ω9	24.168	24.220	24.282	20,22,24	1.781	23.842	.972	24.154	24.163	24.189	24.198

* The list of fatty acid designations in this column was intended to be consistent with the list published by R.G. Ackman (reference 1, cf. tables 4.3A and 4.3B with this table). With the exception of FAMEs 22:2I and 22:2J, the shorthand notation used to identify the fatty acids is described in the text. Ackman has determined that 22:2I and 22:2J are C_{22} non-methylene-interrupted dienoic acids peculiar to marine oils and lipids (reference 1 and references contained therein).

† Within each row, ECL calculations were based on the retentions of the three saturated FAMEs identified in this column using the notation n_1,n_2, and n_3, which refer to the number of carbons in the fatty acid backbone. For example, "14,16,18" would indicate that 14:0, 16:0 and 18:0 were the FAMEs used to determine ECL.

‡ The values for m and b are used for prediction of the ECL for FAMEs at a specific temperature, using the equation of a straight line, as shown in Equation 2.

§ These temperatures are listed to make it convenient to compare ECL values with those of Christie (32) (cf. 175°), Ackman (1) (cf. 180°), Kramer et al. (33) (cf. 195°), and Bannon et al. (31) (cf. 200°).

that can be present in mixtures that contain a marine oil and canola oil. The practice of adding 24:1ω9 to samples to act as an internal standard will probably increase as a result of a recent report (36) that describes its superior performance compared with 23:0. From their analyses at various temperatures (Figure 1), there does not appear to be any temperature in the range 214°C–245°C that would resolve 22:6ω3 and 24:1ω9 while separating 22:5ω3 from 24:0. With this information at their disposal, analysts may want to focus on temperatures either below 214°C or above 245°C. Other separation problems can be handled in a similar way by using more inclusive displays such as those in Figure 3.

With this ability to alter elution patterns by simply rerunning a difficult sample at a different temperature, we have at our disposal a tool to solve problems encountered with complex mixtures. As an example, in Figure 3A, 18:3ω6 elutes before 19:0 below 183°C, whereas above 211°C, it elutes after 19:0. In effect, a run at 183°C and another at 212°C is analogous to making two separations, each with a different liquid phase. This is not a new idea; as far back as 1963, Ackman (37) wrote: "Operation...at a given temperature often will not be completely satisfactory for the detection of some of the components present in complex lipid systems. Use of the same column at different temperatures to resolve components may in many cases be simpler than the use of two different columns."

Coincident peaks caused by addition of internal standards

Whenever possible, it is beneficial to use internal standardization methods in the analysis of fatty acid composition (4,7,16,29,36). This usually involves the addition of one or more odd-chain acids (ideally, as the triacylglycerides) at the very beginning of each analysis (although other fatty acids, such as nervonic acid [24:1ω9], may be more advantageous depending on the circumstances [36]). However, depending on column temperature, the added odd-chain FAME may coincide with polyunsaturated FAME that possesses one fewer carbon: 17:0 with 16:4ω3, 19:0 with 18:3ω6, 21:0 with 20:3ω6, 23:0 with 22:3ω6 (Figure 3). The case of 19:0 and γ-linolenic acid (18:3ω6), described in the previous section, illustrates how such problems can usually be avoided; these esters are not separable between the temperatures of approximately 184°C and 211°C, even on the 60-m capillary column (Figure 3B); those temperatures should be avoided if 19:0 has been added as an internal standard to the samples. Naturally, the same would be true when 19:0 is intrinsic to the sample in more than a trace amount. Caution is also required when 23:0 is used as an internal standard. At any temperature from approximately 203°C to 232°C, its retention time coincides with that of either 22:3ω6 or 21:5ω3 depending on the specific temperature (Figure 3B). A trend toward greater use of internal standards is exemplified by two relatively new official methods for fatty acid composition in fish oils, both of which call for the use of 23:0 as an internal standard: AOAC Method 991.39 (4,5) and AOCS Method Ce-1b-89 (6,7). In the future, 24:1ω9 (36) may be used more often, depending on the sample, and the information presented herein may help the analyst select a temperature to avoid overlaps with that internal standard (Figure 3B).

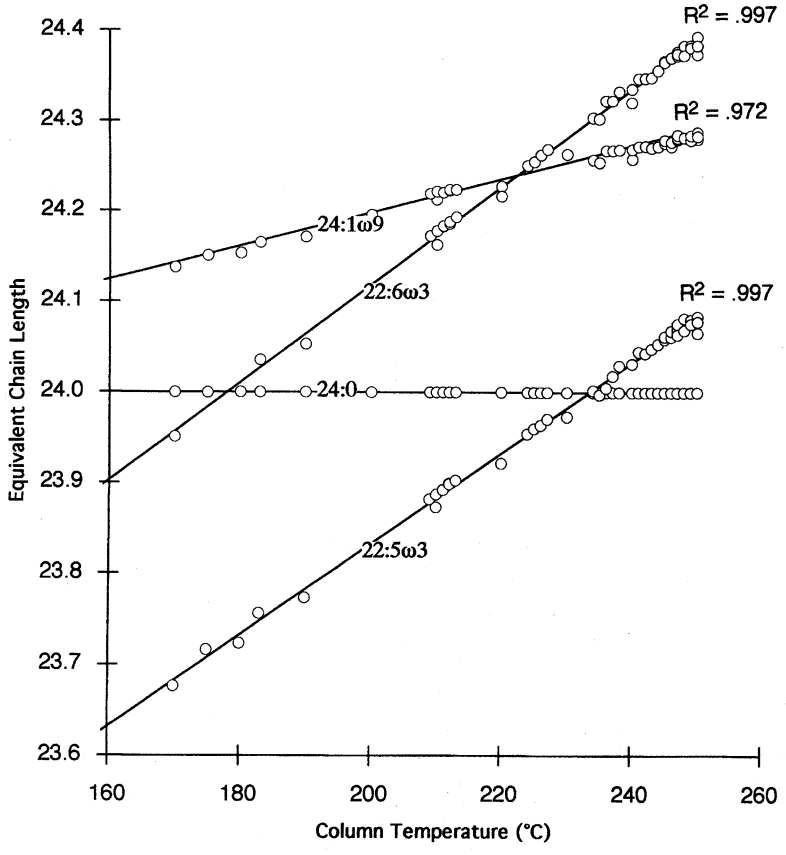

Figure 1. The dependence of ECL on column temperature for four representative FAMEs.

A simple strategy for complex food mixtures

One of the driving forces for this development was the obstacles encountered during the analyses of pooled diet material (diet composites). Due to the variety of components in the diet composites (which contain essentially everything eaten in a day by subjects in diet intervention studies), it was virtually impossible to settle on a temperature programming regime that worked acceptably for all samples in that study. Therefore, only isothermal runs were used, and heavy use was made of ECL information to sort out and identify the multitude of components. Even then, it was found necessary to make more than one chromatographic run for each sample to get consistent results across an entire collection of diet composites.

The ECL temperature database, the creation of which was described previously, was used to select two column temperatures (183 and 212°C) that worked well for the complete analysis of these complicated mixtures (Table I). The FAME of each sample was separated and quantitated in two separate isothermal runs, one at 183°C and a second at 212°C, both on the single column. That

approach circumvented most of the problems caused by coincident peaks, and it provided two independent quantitations of each fatty acid in a given sample, which were useful for quality control purposes.

Run 1 at 183°C

For a majority of fatty acids in each sample, quantitative data and ECL information were obtained in an initial run made at a column temperature of 183°C. A lower temperature could also have been used (to obtain greater detail in the region of the shorter chain esters, if needed) because most of the problems with coincident peaks disappear below 184°C (Figure 3). During the later part of this initial run, at a convenient time for concluding the collection of quantitative data and ECL information (normally following the emergence of 21:0), a temperature program was begun to increase the temperature abruptly to 212°C. By this action, clearance times for longer chain FAMEs were markedly reduced without sacrificing the opportunity to monitor the developing chromatogram for further (qualitative) information about the sample. Meanwhile, the column was becoming established (equilibrated) at the higher temperature in preparation for the second run to follow (at 212°C). The rate of increase used in going from 183°C to 212°C was 10°C/min; a rate above that was too abrupt because it caused 20:3ω6 to accelerate until it became coincident with 21:0 (added as an internal standard); likewise, 22:3ω6 or 21:5ω3 would become coincident with 23:0 (which was also added as a second internal standard). The time required for the first run was only 1 h: approximately 45 min at 183°C isothermal (until emergence of 21:0); approximately 3 min to increase to 212°C at 10°C/min.; and approximately 10 minutes at 212°C isothermal. Quantitation data and ECL information were collected during the entire time at 183°C. Qualitative information about the sample was gathered during and following the rise to 212°C.

Run 2 at 212°C

As a result of the actions described in the previous paragraph, the instrument was already equilibrated for a second run, which would be made at 212°C and could begin without delay. In addition to providing a second set of ECL values for comparison, increasing sensitivity (better integrator performance), and improving peak shapes for longer chain esters (only very slightly skewed at 183°C), the second step provided a second set of values, which were useful for comparison purposes, on the levels of fatty acids. Table I shows ECL for both 183°C and 212°C.

Efficient use of time

There was a penalty in terms of time for the double-run procedure just described, but it was small, and it was minimized by the following procedures. Before continuing with the analyses, the column temperature was set to the highest temperature of the two temperatures to be used, normally 212°C. Then the linear velocity of carrier gas (μ) was adjusted to its optimum, approximately 40 cm/s for hydrogen (21,24,28). Then the temperature was lowered to 183°C, and the first sample was run without readjustment of μ. The reduction in temperature to 183°C caused a concomitant increase in μ (owing to the decrease in gas density), but it was not enough to significantly degrade the separations. When the temperature was raised again to 212°C, the optimal μ was already set.

There were several benefits derived from choosing hydrogen instead of helium as the carrier gas for these separations. Hydrogen gave separations virtually equivalent to helium (21,25,27) but in less time, which made it practical to use the 60-m column instead of a 30-m version. The use of helium in a 60-m column resulted in prohibitively long retention times for the longer chain FAME. Also, as can be seen in plots of HETP versus carrier gas linear velocity with helium and hydrogen (24,27), the use of hydrogen results in far less diminution in column resolution when operating at a velocity that is higher than the point of peak resolution. In practice, this means that chromatographers can proceed from their first analysis at 183°C directly to the second at 212°C without readjustments to the carrier gas supply settings.

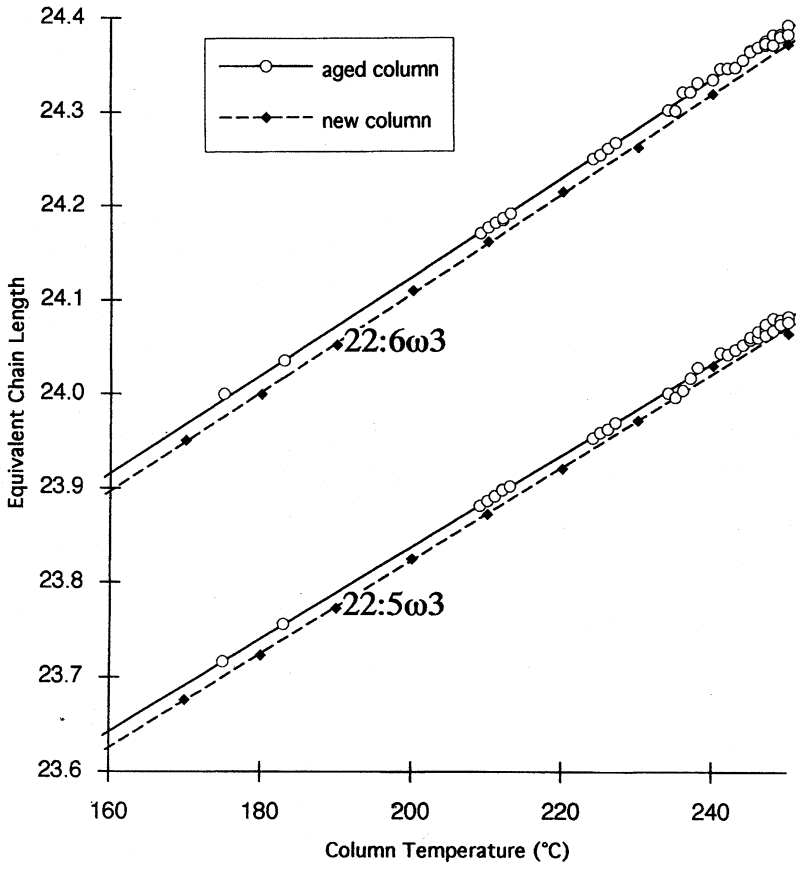

Figure 2. Comparison of ECL values and regression results obtained with a new column and with the same column after a period of approximately 10 days at or above 180°C.

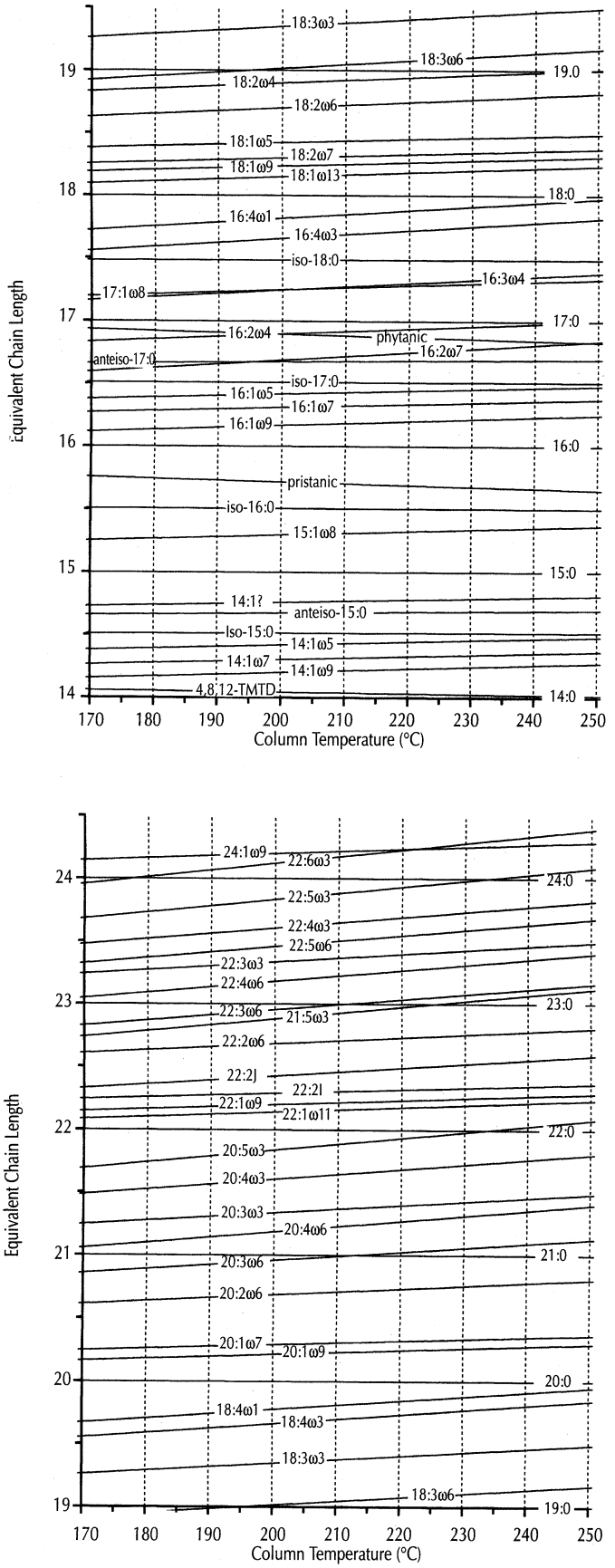

Figure 3. Graph for prediction of retention times for FAME with ECL values (A) between 14.0 and 19.5 and (B) between 19.0 and 24.5 at specific column temperatures.

Conclusion

In this laboratory, in the analyses of complicated mixtures of food materials, the additional time needed to make two runs per sample (isothermal, different temperatures) instead of just one with the use of some sort of temperature program has always been recaptured because of time saved in the interpretation of chromatograms. Interpretation can be tedious, aggravating, and labor- intensive, but a double-run strategy can be done automatically with modern instrumentation under programmed instrument control. The total time for the double-run approach was not unreasonable; the first run required not more than 1 h, and the second required not more than 45 min. In addition, many of the foods that were analyzed had simple FAME patterns, so analysis was accomplished with a single separation at 183°C.

The double-run strategy becomes more important with complicated mixtures of food materials, where the additional ECL information aids analysts in interpretation, quality control, and reporting. All FAME components are usually well-separated on the 60-m column, either at the first temperature or the second or both. By taking advantage of the shift in polarity with a change in temperature, it was as though the second run had been made with a different liquid phase. Two full sets of ECL values were easily derived for the FAME in each sample; in addition, there were two sets of values on the levels of each FAME in the sample.

Obviously, 183°C and 212°C were not the only choices available for column temperature, but the choices were surprisingly limited. As can be seen in Figure 3B, any temperature between 213°C and 247°C was excluded because of problems with the separation of some important critical pairs of FAMEs, particularly for 22:6ω3 and 24:1ω9 and for 22:5ω3 and 24:0. Temperatures above 247°C showed promise for analysis of longer chain FAMEs, particularly those eluting beyond 20:0; all were well-separated, and the analysis was completed in a very short time. Unfortunately, sustained operation at that high temperature substantially reduced the useful life of these columns; this will be the subject of additional research.

The author essentially agrees with several premises offered by Krupcik and Bohov (35): ECL values can be used for tentative identification of FAMEs only when they are chromatographed under isothermal conditions;

isothermal capillary GLC of FAMEs is tedious in analytical practice; and isothermal analyses do not resolve all FAMEs, which has resulted in their analysis most often under temperature-programmed conditions. Those authors took the approach of developing mathematical and graphical treatments that allowed them to characterize FAMEs using ECL information taken from linear temperature-programmed runs (35). The tactic that has worked best in this laboratory is to make a second tedious run at another temperature.

Errors can arise in either quantitative or qualitative analysis when there are artifacts present in the sample. For this reason, one class of compounds in particular, namely the phthalic acid esters, should be included in the accompanying ECL compilation. Fortunately, according to Ackman (personal communication, 1995), these ubiquitous artifacts show a radical shift in ECL with temperature change; therefore, the use of two temperatures will be advantageous in avoiding mistakes when analyzing food materials contaminated with phthalates. These topics have been thoroughly covered by Shantha and Ackman (38), who have shown that the original phthalic acid esters (i.e., those forms initially occurring as a result of the addition of plasticizers) are not the only forms that result in difficulties; other mixed alcohol esters of phthalic acid are produced in the transmethylation step in preparation for GLC, and some of them co-chromatograph with FAMEs (depending on the column temperature).

Nutrition labeling was a major impetus for this effort. We aimed at the development of a more standardized approach to routine fatty acid analyses. Since January 1993, food labeling regulations in the United States have called for "fat" to be calculated from available fatty acid data (sum of fatty acids as triacylglycerols) (39). Consequently, the amount of each individual fatty acid must be determined, either by chemical analysis or by reference to approved databases. This concept of "fat" makes it imperative that all the fatty acids be accurately identified and quantitated and that artifacts not be accidentally included.

Because the *trans*- and *cis*- isomers of FAMEs are not sufficiently well-separated on this liquid phase (cross-linked Carbowax-20M), the method discussed herein did not provide anything new in terms of their analysis in food extracts; this result was expected. At present, these compounds are best handled with long capillary columns, at least 100 m in length, prepared with a polar liquid phase (16,20,40–44). Another alternative may be to use the polar liquid phase in a shorter, 60-m column and couple those results with a separate infrared analysis, as is done in the new AOCS method (Cd-14b-93) for margarines. A different, but also comparatively recent, AOCS method (Ce-1c-89) relies on GLC alone with 60-m columns, and it has been criticized for causing underestimation of *trans* isomer content (45–47); this weakness results from the inability of the shorter column (60-m versions) to sufficiently resolve the isomeric octadecenoates.

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